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Citation for published version:

Elsberger, B, Tan, BA, Mitchell, TJ, Brown, SBF, Mallon, EA, Tovey, SM, Cooke, TG, Brunton, VG & Edwards, J 2009, 'Is expression or activation of Src kinase associated with cancer-specific survival in ER-, PR- and HER2-negative breast cancer patients?', *The American Journal of Pathology*, vol. 175, no. 4, pp. 1389-97. <https://doi.org/10.2353/ajpath.2009.090273>

Digital Object Identifier (DOI):

[10.2353/ajpath.2009.090273](https://doi.org/10.2353/ajpath.2009.090273)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

The American Journal of Pathology

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Biomarkers, Genomics, Proteomics, and Gene Regulation

Is Expression or Activation of Src Kinase Associated with Cancer-Specific Survival in ER-, PR- and HER2-Negative Breast Cancer Patients?

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The aim of the current study was to assess the expression levels of c-Src and phosphorylated Src kinase in human breast cancers and to establish if these are linked to oestrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 status or patient survival. Tissue microarray technology was used to analyze 314 breast cancer specimens. Immunohistochemistry was performed using antibodies to c-Src, Y419Src, and Y215Src, and expression was assessed using the weighted histoscore method. High cytoplasmic c-Src kinase and high membrane phosphorylated activated Y419Src kinase was associated with decreased disease-specific survival. In contrast, phosphorylated activated nuclear and cytoplasmic Y215Src kinase expression levels were significantly associated with improved disease-specific survival. When the cohort was subdivided according to ER/PR/HER2 status, the ER-negative subgroup (105 patients) was associated with improved disease-specific survival and was found to be independent by multivariate analysis with a hazard ratio of 0.4 (interquartile range 0.2–0.8). High cytoplasmic c-Src expression was associated with decreased survival; high expression of activated c-Src (Y215) was associated with improved survival. This was potentiated in the ER/HER2-negative subgroup. Hence, administration of Src kinase inhibitors aiming to decrease phosphorylation should be approached with caution, especially in ER-negative patients. It is therefore essential to appropriately identify with the correct biomark-

ers which patients are most likely to respond to Src inhibitors. (Am J Pathol 2009, 175:1389–1397; DOI: 10.2353/ajpath.2009.090273)

The nonreceptor tyrosine kinase, c-Src, is implicated as a regulator of cell proliferation and survival and has a complex role in cell adhesion, proliferation, and motility.¹ *In vitro* work has implicated c-Src in the development and progression of human breast carcinoma.^{2,3} However, there is little evidence to support this observation in clinical specimens.

c-Src is composed of a C-terminal tail, kinase domain, two protein-protein interaction domains (SH2, SH3) and a unique amino-terminal domain that varies between Src family members. c-Src is activated by a number of pathways. First, dephosphorylation of Y530 by a number of phosphatases has been linked to activation. Second, the binding of the SH2 and SH3 domains to various proteins (epidermal growth factor receptor, human epidermal growth factor receptor 2 (HER2), fibroblast growth factor receptor, focal adhesion kinase (FAK), p130^{CAS} (4–6)) is thought to be important in c-Src regulation. This direct association can result in activation of the intrinsic tyrosine kinase activity of Src and/or localization of Src to sites of action. For example, platelet-derived growth factor or HER2-driven phosphorylation of c-Src at Y215 (SH2 domain) has been shown to block binding along with the C-terminal regulatory sequence resulting in a 50-fold activation of Src.⁷

Investigating the role of the Src kinase in breast cancer at each activated phosphorylation site (Y215, Y419) along with the total expression levels of the protein is required to determine its significance. The site of Src expression within the

Supported by Royal College of Surgeons Edinburgh.

Accepted for publication July 2, 2009.

Sadly during the preparation of this manuscript our colleague and friend Professor Timothy Cooke died suddenly. We wish to acknowledge his support through the development and execution of this study.

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cell should also be assessed. Classically, the association of c-Src with the membrane is considered essential for cellular transformation;⁸ however, the location of activated Src protein in breast cancer is unclear in the literature^{2,9,10} with only one report of membranous staining. It has been suggested that cytosolic Src functions for protein trafficking, and there is early intriguing data suggesting a role for nuclear Src as a cell cycle regulator.¹¹

Although cell line studies strongly support the role of c-Src in breast cancer progression, translational studies investigating human breast tumor expression and correlation with expression and activation to clinical parameters are surprisingly limited.^{2,3} There are currently few published studies with data on expression of Src kinase in clinical breast cancer specimens.

The hypothesis of this study is that c-Src expression and or phosphorylation status is linked with decreased breast cancer-specific survival. The aim of this study was therefore to assess the expression levels of c-Src and activated Src (at two different phosphorylation sites) in human breast cancers and determine any correlation with patient outcome measures.

Materials and Methods

Patients

A total of 314 patients were recruited. All patients were diagnosed with operable invasive breast carcinoma between 1980 and 1999 in the Greater Glasgow area. These patients received standard adjuvant treatment according to protocols at the time of diagnosis. We only included patients in our analysis when all clinical data, ER, PR and HER2 status and full Src kinase and activated Src kinase expression data, were available. Ethics approval was granted by the local ethics committee.

Tissue Microarray Construction

Tissue microarrays (TMAs) were already available for use in the current study, 0.6 mm² cores of breast cancer tissue, identified by the pathologist, were removed from representative areas of the tumor taken from breast cancer patients at time of surgical resection. All TMA blocks were constructed in triplicate.

Immunohistochemistry

Staining for ER, PR, and HER2 had been previously performed for the cohort.¹² Fluorescent *in situ* hybridization was performed on tumors that scored 2+ by the HERCEPT test (DakoCytomation, Glostrup, Denmark). Tumors were deemed as HER2 positive if scored 3+ by HERCEPT test or if 2+ by HERCEPT test and amplified by fluorescent *in situ* hybridization using the Pathvision test (Vysis, USA). Src kinase and activated Src kinase expression (Y419Src and Y215Src) was investigated using antibodies for c-Src (36D10; Cell Signaling Technology, MA), Y416Src (Cell Signaling Technology, Beverly, MA), and Y216Src (Santa Cruz Biotechnology, Santa Cruz, CA). In humans, the two acti-

vating phosphorylation sites that were investigated in the current study are amino acids Y215 and Y419. Antibodies used relate to the rabbit sequence and not the human sequence. Tissue sections were dewaxed and rehydrated through graded alcohol. All three antibodies were incubated in a preheated antigen retrieval solution (citrate buffer, pH 6.0; Vector Laboratories, Burlingame, CA). Antigen retrieval was performed by heating tissue sections under pressure for 5 minutes in a microwave. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide (H₂O₂) (c-Src and Y416Src) and 3% H₂O₂ solution (Y216Src). To reduce nonspecific binding, the tissue sections were then incubated with 1.5% normal horse serum (c-Src) and 5% normal horse serum (Y416Src and Y216Src) (Vector Laboratories) in antibody diluent (DakoCytomation) for 20 minutes at room temperature. Slides were incubated with c-Src (1/200 dilution, 4.32 µg/ml) for 60 minutes at room temperature. The phosphorylated antibodies (Y419Src, dilution 1/2000, 0.02 µg/ml; and Y216Src, dilution 1/25, 8 µg/ml) were incubated overnight at 4°C. Signal was amplified and visualized using the DAKO Envision Kit (DakoCytomation) and the chromagen 3,3'-diaminobenzidine (Vector Laboratories). Sections were counterstained, dehydrated, and mounted. In each run, a positive and negative isotype-matched control was included to ensure no false-positive staining or intense stromal staining. Specificity of all antibodies was confirmed by Western blotting.

Scoring

Protein expression of each core (three per tumor specimen) was assessed using the weighted histoscore method (H score method,¹³). The weighted histoscore grades staining intensity as negative (0), weak (1), moderate (2), and strong (3), then multiplication of the percentage of tumor cells within each category. The histoscore ranged from zero (minimum) to 300 (maximum). Agreement between observers was excellent and measured in interclass correlation coefficient, respectively. All interclass correlation coefficient scores were above 0.84.

Statistical Analysis

Disease-specific survival rates were generated using the Kaplan-Meier method. The log-rank test was used to compare significant differences between subgroups using univariate analysis. On the basis of the results of the univariate analysis, a multivariate analysis was then performed. The multivariate stepwise Cox-regression analysis was performed to identify factors that were independently associated with disease-specific death. A stepwise backward procedure was used to derive a final model of the variables that had a significant independent relationship with survival. To remove a variable from the model the corresponding *P* value had to be >0.05.

Interrelationships between clinical parameters and ER, PR and HER2 status were calculated using the χ^2 test. Because of the number of statistical comparisons, a value of *P* < 0.01 was considered to be significant. Data are expressed as median and range. The statistical anal-

Table 1. The Interrelationships between the Clinicopathological Characteristics of Patients with Breast Cancer

Total patient cohort: 314		χ^2 P values															
Variables	Numbers	Tumour type	Grade	Size	LN status	ER status	PR status	HER status	c-Src nuc	c-Src cyto	c-Src memb	Y419Src nuc	Y419Src cyto	Y419Src memb	Y215Src nuc	Y215Src cyto	Y215Src memb
Age (<50 yr/ >50 yr)	78/236	0.910	0.728	0.261	0.950	0.260	0.947	0.173	0.527	0.369	0.705	0.622	0.391	0.287	0.332	0.649	0.861
Tumour type (duct/lob/ tub/others)	298/10/3/3		0.007	0.964	0.680	0.012	0.453	0.512	0.904	0.717	0.761	0.753	0.293	0.416	0.015	0.515	0.424
Grade (G1/ G2/G3)	22/146/146			<0.001	0.301	<0.001	<0.001	0.003	0.019	<0.001	0.017	0.627	0.069	<0.001	0.006	0.674	0.372
Size (mm) (<20, 20– 50, >50)	126/169/19				<0.001	0.048	0.169	0.010	0.695	0.177	0.029	0.061	0.044	0.002	0.061	0.921	0.399
Lymph node (positive/ negative)	152/162					0.555	0.812	0.016	0.937	0.329	0.607	0.127	0.211	0.392	0.504	0.828	0.217
ER status (positive/ negative)	209/105						<0.001	<0.001*	0.037	<0.001*	0.001*	0.143	0.108	<0.001*	<0.001	0.902	0.972
PR status (positive/ negative)	149/163							0.001	0.237	<0.001	<0.001	0.412	0.042	<0.001	0.014	0.266	0.087
HER2 status (positive/ negative)	51/263								0.031	<0.001	0.001	0.053	<0.001	<0.001	0.161	<0.001†	0.075
c-Src nuc (positive/ negative)	139/175									<0.001	<0.001	0.058	0.268	0.376	0.902	0.297	0.048
c-Src cyto (positive/ negative)	139/175										<0.001	0.687	<0.001	<0.001	0.745	0.009*	0.682
c-Src memb (positive/ negative)	135/179											0.370	0.001	<0.001	0.147	<0.001*	0.054
Y419Src nuc (positive/ negative)	122/192												<0.001	0.004	0.042	0.005*	0.120
Y419Src cyto (positive/ negative)	138/174													<0.001	0.294	<0.001*	0.489
Y419Src memb (positive/ negative)	153/161														0.690	<0.001*	0.141
Y215Src nuclear (positive/ negative)	154/160															<0.001	0.063
Y215Src cyto (positive/ negative)	158/156																0.022
Y215Src memb (positive/ negative)	14/299																

Histology: duct, ductal carcinoma; lob, lobular carcinoma; tub, tubular carcinoma, others including mucinous, mucoid and micropapillary carcinoma; Grade, Bloom and Richardson grade; c-Src, total Src kinase; Y419Src, Src kinase phosphorylated at tyrosine site 419; Y215Src, Src kinase phosphorylated at tyrosine site Y215; c-Src nuc, c-Src nuclear expression; c-Src cyto, c-Src cytoplasmic expression; c-Src memb, c-Src membrane expression; Y419Src nuc, Y419Src nuclear expression; Y419Src cyto, Y419Src cytoplasmic expression; Y419Src memb, Y419Src membrane expression; Y215Src nuc, Y215Src nuclear expression; Y215Src cyto, Y215Src cytoplasmic expression; Y215Src memb, Y215Src membrane expression. *, Negatively correlated; †, cytoplasmic Y215Src kinase expression independent on multivariate analysis ($P = 0.005$, Table 3) as HER2 being displaced from the Cox regression model.

yses were performed using a statistical software package (version 15.0; SPSS, Chicago, IL).

Results

Clinicopathological Details

Our full cohort consisted of 314 breast cancer patients (209 ER positive/105 ER negative and 149 PR positive/163 PR

negative) (Table 1; bold typeface used to highlight significant results). Median age was 58 years (interquartile range 51–56). Forty-six percent of the cancer specimens were pathologically graded as grades 2 and 3, median size of the invasive cancer was 20 mm (IQR 15–30 mm). Forty-eight percent of the patients were axillary lymph node positive. Mean patient follow-up was 7.1 years (minimum follow-up was 2.1 years and the maximum follow-up was 20 years). Sixteen patients were lost to follow-up. During this period,

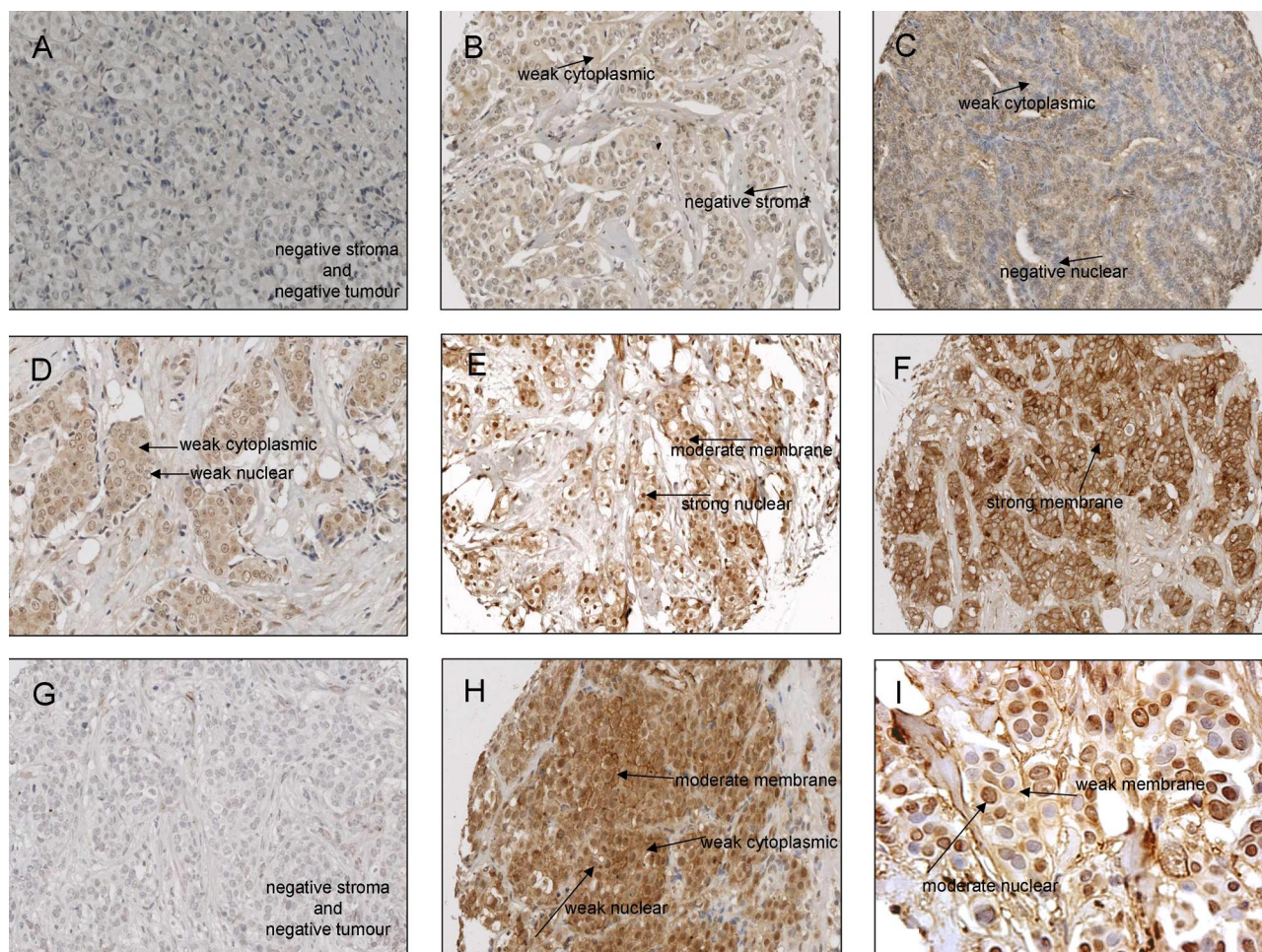


Figure 1. Images of immunohistochemistry (IHC) for each antibody. **A–C** shows breast tissue stained with c-Src antibody (1:200; Cell Signaling Technology); **A** demonstrates negative stroma and negative breast tumor staining; magnification, $\times 100$; **B** illustrates weak cytoplasmic and negative stroma staining; magnification, $\times 100$; **C** displays negative and weak cytoplasmic staining as well as negative and weak nuclear staining; magnification, $\times 60$. **D–F** shows breast tissue stained with Y416Src antibody (1:2000; Cell Signaling Technology); **D** reveals weak cytoplasmic and weak nuclear staining; magnification, $\times 100$; **E** demonstrates strong nuclear and moderate membrane staining; magnification, $\times 40$; **F** displays strong membrane staining; magnification, $\times 60$. **G–I** shows breast tissue stained with Y216Src antibody (1:25; Santa Cruz Biotechnology); **G** illustrates negative staining of stroma and tumor tissue; magnification, $\times 100$; **H** reveals weak cytoplasmic, weak nuclear and moderate membrane staining; magnification, $\times 100$; **I** demonstrates negative cytoplasmic, moderate nuclear and weak membrane staining; magnification, $\times 400$.

78 patients died of their cancer, and an additional 33 patients died of intercurrent disease. Correlations between the clinicopathological characteristics of this cohort are shown in Table 1.

Src Kinase Expression Levels

c-Src kinase

Each cellular location was independently assessed for c-Src kinase expression levels. Forty-five percent of tumors exhibited nuclear expression, 60% cytoplasmic, and 43% membrane (Figure 1, A–C). Tumors were subdivided into those with high (above the median) or low (below or equal to the median) expression. χ^2 analysis demonstrated that grade and HER2 status positively correlated with cytoplasmic c-Src expression (Table 1). ER status correlates negatively with cytoplasmic and membrane c-Src expression (Table 1). c-Src expression in the membrane only correlates with HER2 status (Table 1).

Expression levels of c-Src at each cellular location positively correlated with each other (Table 1). On univariate analysis, neither membrane nor nuclear c-Src expression was associated with disease-specific survival. Whereas high cytoplasmic c-Src kinase expression was significantly associated with shorter disease-specific survival (P 0.028) (Figure 2A), but was not independent in multivariate analysis (Table 2; bold typeface used to highlight positive correlations). Those patients with high cytoplasmic c-Src expression had a median survival of 12.6 years (IQR 10.8–14.5) compared with those with low expression with median survival of 14.6 years (IQR 12.8–16.5).

Y419Src kinase

Activated phosphorylated Src kinase expression, at the classical activation site Y419, was predominately observed in the nucleus (92%) and cytoplasm (76%) and

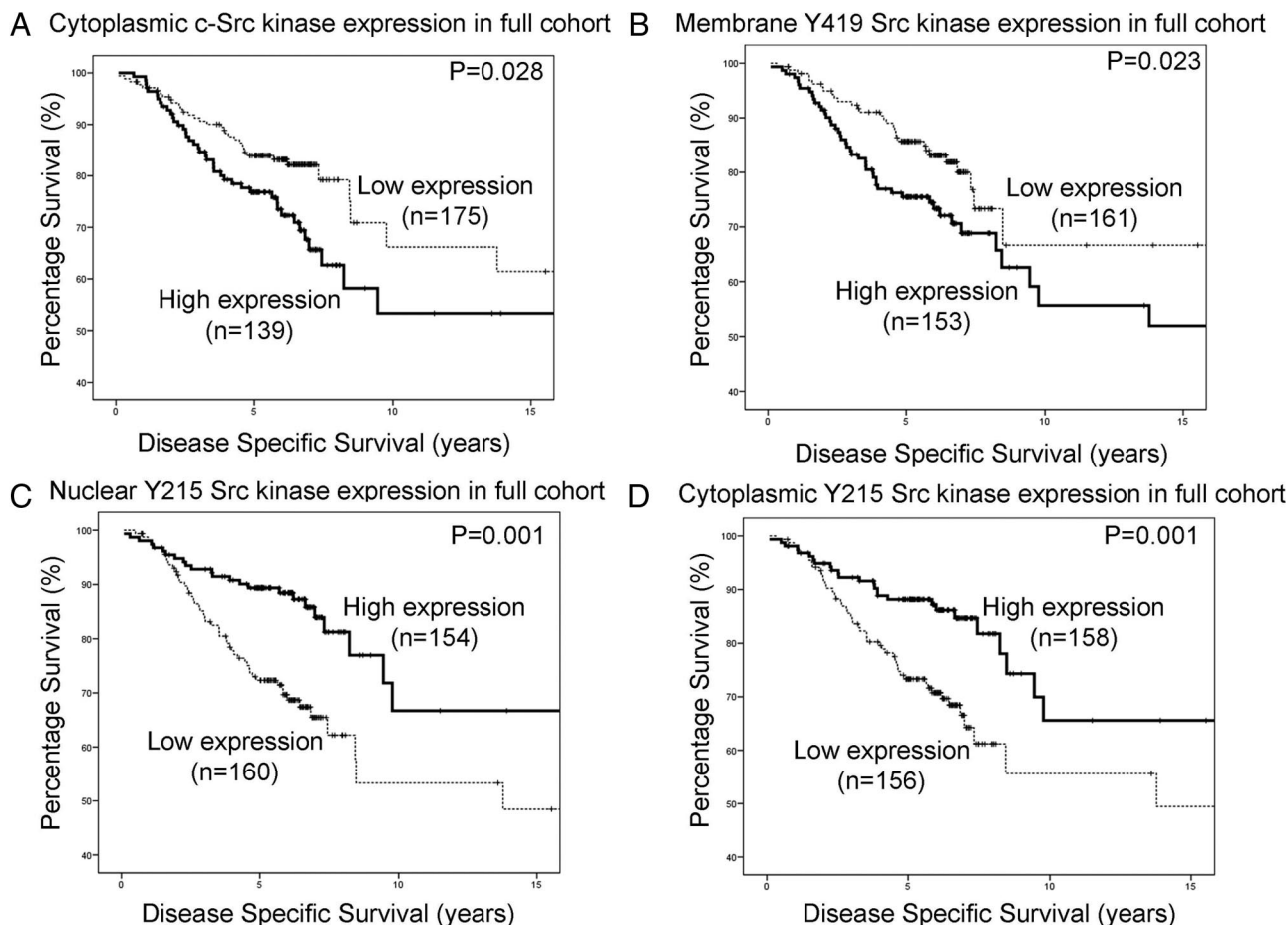


Figure 2. Kaplan-Meier Curves for Src kinase expressions in the full cohort ($n = 314$). **A:** Kaplan-Meier Survival Curve cytoplasmic c-Src ($P = 0.028$). **B:** Kaplan-Meier Survival Curve membranous Y419Src ($P = 0.023$). **C:** Kaplan-Meier Survival Curve nuclear Y215Src ($P = 0.001$). **D:** Kaplan-Meier Survival Curve cytoplasmic Y215Src ($P = 0.001$).

48% of tumors also expressed Y419Src kinase in the membrane (Figure 1, D–F). χ^2 analysis demonstrated that cytoplasmic Y419Src expression positively correlated with HER2 status (Table 1). Membrane Y419Src expression positively correlated with grade, size, and HER2 status and inversely correlated with ER status (Table 1). Nuclear Y419Src expression did not correlate with any known clinicopathological features. Expression levels of Y419Src at each cellular location strongly positively correlated with each other (Table 1). Only high membrane expression was associated with shorter disease-specific survival ($P = 0.023$) on univariate analysis (Figure 2B) but was not independent in multivariate analysis (Table 2). Patients with high membrane Y419Src expression had a median survival of 12.8 years (IQR 11.1–14.6) compared with those with low expression with median survival of 14.8 years (IQR 12.1–16.8).

Y215Src kinase

Phosphorylated activated Y215Src kinase expression was predominately observed in the nucleus (74%) and cytoplasm (68%) (Figure 1, G–I). No more than 5% of

tumors expressed Y215Src kinase in the membrane. On χ^2 analysis, nuclear Y215Src expression positively correlated with grade and ER status (Table 1). The only correlation observed with cytoplasmic Y215Src expression and clinicopathological features was a positive correlation with HER2 status (Table 1).

In contrast, to the observations made with total c-Src (associated with poor prognosis), high nuclear and cytoplasmic Y215Src kinase expression was strongly associated with improved disease-specific survival (nuc Y215Src, $P < 0.001$ (Figure 2C); cyto Y215Src, $P = 0.001$ (Figure 2D)) (Table 2). Although cytoplasmic Y215Src kinase expression and HER2 status correlated on χ^2 analysis (Table 1), only cytoplasmic Y215Src kinase expression was independent on multivariate analysis ($P = 0.005$) with a hazard ratio 0.5 (IQR 0.3–0.8), as HER2 was displaced from the Cox-regression model. Patients with high nuclear Y215Src kinase expression had a median survival of 15.0 years (IQR 13.1–16.8) compared with those with low expression with a median survival of 12.3 years (IQR 10.5–14.2). Patients with high cytoplasmic Y215Src kinase expression had a median survival of 14.9 years (IQR 13.1–16.7) compared with those

Table 2. Impact of Clinicopathological Factors and Protein Expression/Activation on Patient Survival

Total patient cohort: 314 patients	Univariate analysis				Multivariate analysis		
	Numbers	<i>P</i> value	HR	IQR	<i>P</i> value	HR	IQR
Age (<50 yr/>50 yr)	78/236	0.351	1.2	0.7–2.2			
Tumour type (ductal/lobular/tubular/others)	298/10/3/3	0.714	0.6	0.2–1.4			
Grade (G1/G2/G3)	22/146/146	0.014	1.8	12–2.7			
Size (<20 mm, 20–50 mm, >50 mm)	126/169/19	<0.001	3.1	2.1–4.8	<0.001	6.7	3.2–14.2
Lymph node (positive/negative)	152/162	0.001	2.1	1.3–3.3	0.013	1.9	1.1–3.1
ER status (positive/negative)	209/105	<0.001	2.3	1.5–3.6	0.027	1.8	1.1–3.0
PR status (positive/negative)	149/163	0.006	1.9	1.2–3.0	0.04	1.7	1.0–2.7
HER2 status (positive/negative)	51/263	0.010	1.9	1.6–3.2			
c-Src nuclear expression (positive/negative)	139/175	0.328	1.2	0.7–2.0			
c-Src cytoplasm expression (positive/negative)	139/175	0.028	1.6	1.1–2.6			
c-Src membrane expression (positive/negative)	135/179	0.784	0.94	0.6–1.5			
Y419Src nuclear expression (positive/negative)	122/192	0.585	0.87	0.5–1.4			
Y419Src cytoplasm expression (positive/negative)	138/174	0.625	0.88	0.6–1.4			
Y419Src membrane expression (positive/negative)	153/161	0.023	1.7	1.1–2.7			
Y215Src nuclear expression (positive/negative)	154/160	<0.001	0.43	0.2–0.7			
Y215Src cytoplasm expression (positive/negative)	158/156	0.001	0.45	0.3–0.7	0.005	0.5	0.3–0.8
Y215Src membrane expression (positive/negative)	14/299	0.601	0.74	0.2–2.3			

Table 2 shows an overview of the full patient cohort's characteristics. Each clinical and pathological parameter was correlated to disease-specific survival (*P* values). Grade, Bloom and Richardson grade. Histology: ductal, ductal carcinoma; lobular, lobular carcinoma; tubular, tubular carcinoma; others including mucinous, mucoid, and micropapillary carcinoma c-Src, total Src kinase; Y419Src, Src kinase phosphorylated at tyrosine site 419; Y215Src, Src kinase phosphorylated at tyrosine site Y215.

with low expression with a median survival of 12.5 years (IQR 10.6–14.4).

Relationship of Src Kinase Expression to Disease-Specific Survival in Patients Stratified by ER Status

Src kinase and activated Src kinase expression was then stratified by ER status (105 ER-negative and 209 ER-positive tumors). In patients with ER-negative tumors, no significant associations with survival were made with membrane, cytoplasmic, or nuclear c-Src expression. However, cytoplasmic Y215Src kinase expression was significantly associated with improved disease-specific survival (*P* = 0.003) (Figure 3A) and was, as in the full cohort, independent on multivariate analysis (*P* = 0.007) with a hazard ratio of 0.4 (IQR 0.2–0.8). No association was observed in the ER-positive cohort (*P* = 0.051) (Figure 3B). In contrast, it was nuclear Y215 Src kinase expression that was significantly associated with improved disease-specific survival (*P* = 0.003) (Figure 3D) in the patients with ER-positive tumor, and this was also independent on multivariate analysis (*P* = 0.009) with a hazard ratio of 0.4 (IQR 0.2–0.8). No association was observed in the ER-negative cohort (*P* = 0.345) (Figure 3C).

When patients with ER/HER2-negative tumors were analyzed (*n* = 75), cytoplasmic Y215 Src kinase was yet again strongly associated with improved disease-free

survival (*P* = 0.007) (Figure 4), and this was independent on multivariate analysis (*P* = 0.040) with a hazard ratio of 0.3 (IQR 0.1–0.9) (Table 3; bold typeface used to highlight significant results). This observation held when ER/PR/HER2-negative tumors were analyzed (*n* = 71) (univariate analysis *P* = 0.015, multivariate analysis *P* = 0.021).

Discussion

This study suggests that Src kinase expression and phosphorylation status may be used as prognostic markers in breast cancer. Increased cytoplasmic c-Src kinase expression is significantly associated with decreased disease-specific survival. To our knowledge, this is currently the largest study investigating the role of Src kinase expression and phosphorylation status in a cohort of clinical breast cancer specimens. Most of the existing *in vitro* literature provides compelling evidence that c-Src activation is associated with high tumor proliferation, increased invasion, and increased migration in breast cancer cells.^{1,14} This has been supported by an *in vivo* study where Src kinase expression in ductal carcinoma *in situ* breast tumors correlated with HER2 positivity, high tumor grade, comedo necrosis, and elevated epithelial proliferation.³ In addition, high c-Src levels were associated with lower recurrence-free survival. The results obtained from our study are in keeping with those cell line studies,

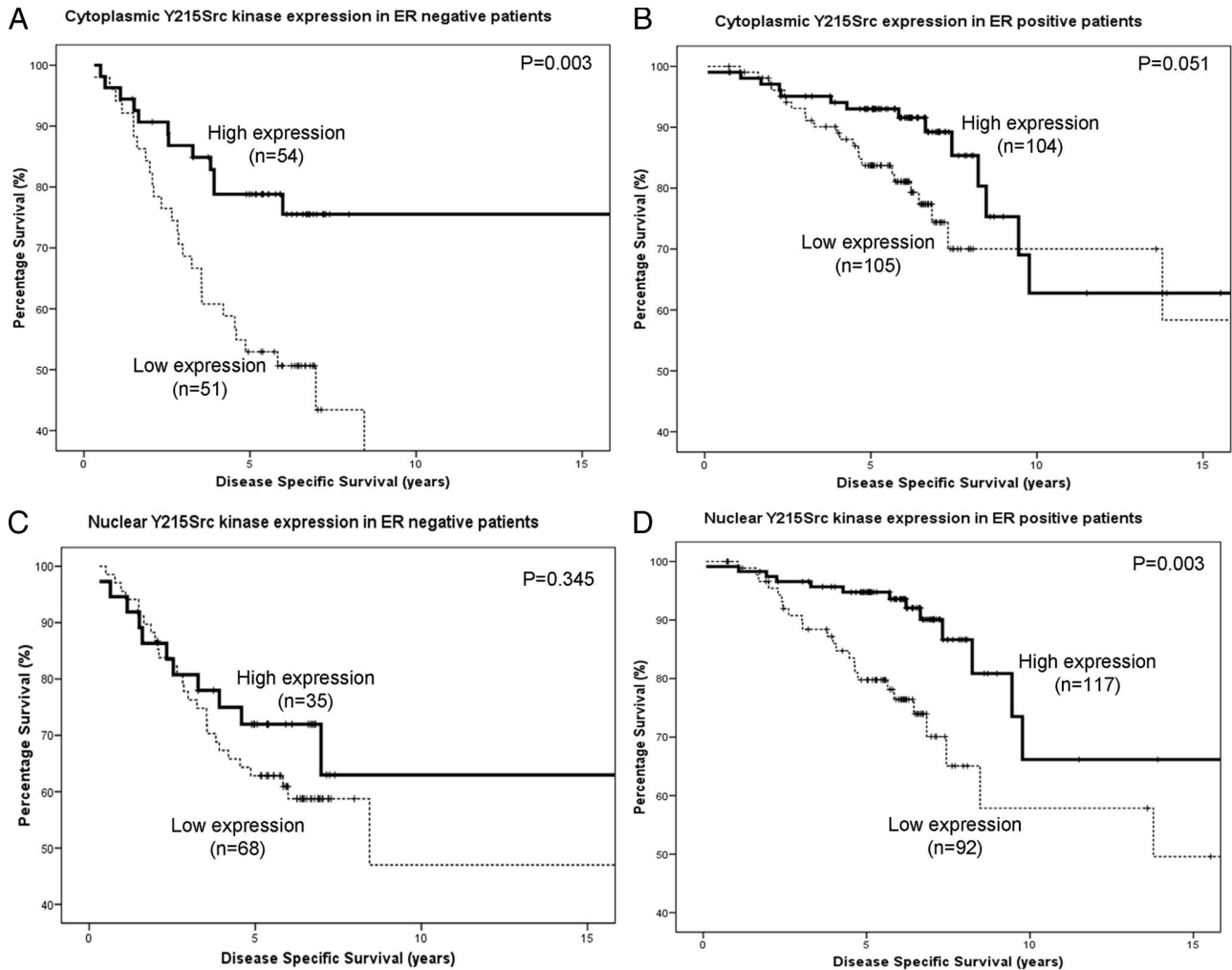


Figure 3. Kaplan-Meier Survival Curves for Y215Src expression for ER positive ($n = 209$) and negative ($n = 105$) subgroups. **A:** Kaplan-Meier Survival Curve for nuclear Y215Src in ER-negative patients ($P = 0.345$). **B:** Kaplan-Meier Survival Curve for nuclear Y215Src in ER-positive patients ($P = 0.003$). **C:** Kaplan-Meier Survival Curve for cytoplasmic Y215Src in ER-negative patients ($P = 0.003$). **D:** Kaplan-Meier Survival Curve for cytoplasmic Y215Src in ER-positive patients ($P = 0.051$).

demonstrating that c-Src is associated with more aggressive growth and poor clinical outcome. Confirmed in the current study, c-Src expression correlated with

decreased survival, increased tumor grade, and ER and HER positivity. Although it is important to assess c-Src protein levels, it is of equal importance to determine the activation status of Src kinase. Ito et al.² reported that high activated c-Src expression was frequently observed in breast tumors with lower aggressiveness as indicated by low Ki-67, absence of lymph node spread, small size, and a low histological grade. This suggests that activation of Src is associated with early stages of breast carcinoma, but there was no correlation made with patient outcome measures.² However, Src activation was determined using an antibody to tyrosine site 530 and was classed as activated if unphosphorylated at this site. It has previously been suggested that a more appropriate biomarker for prediction of clinical response to Src kinase inhibitors would be to measure phosphorylation of the protein at a site associated with activity.^{15,16} Currently, there are two sites within c-Src known to be associated with activation. Y419Src is known as the classical site, which is most commonly used in cell line studies investigating the functional relevance of Src kinase activation.¹⁷ Src kinase when activated is moved to the mem-

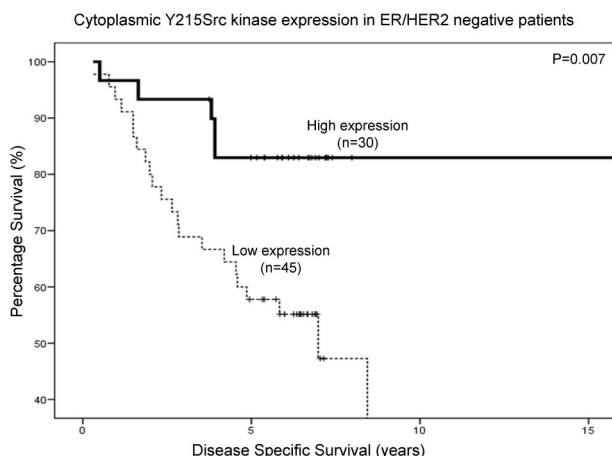


Figure 4. Kaplan-Meier Survival Curve for cytoplasmic Y215Src in ER/HER2-negative patients ($P = 0.007$).

Table 3. Impact of Clinicopathological Factors and Protein Expression/Activation on Patient Survival in ER/HER2-Negative Patients

ER/HER2-negative patients: 75 patients			Univariate analysis		Multivariate analysis		
	Numbers	P value	HR	IQR	P value	HR	IQR
Grade (G1/G2/G3)	1/13/61	0.214	2.0	0.9–4.5			
Size (<20 mm, 20–50 mm, >50 mm)	31/37/7	<0.001	5.2	1.9–14.7	0.030	5.1	1.7–15.0
Lymph node (positive/negative)	35/40	0.021	2.4	1.1–5.4	0.005	3.3	1.4–7.5
c-Src nuclear expression (positive/negative)	39/36	0.747	1.1	0.5–2.4			
c-Src cytoplasm expression (positive/negative)	39/36	0.412	1.4	0.6–3.0			
c-Src membrane expression (positive/negative)	36/39	0.636	1.2	0.6–2.6			
Y419Src nuclear expression (positive/negative)	17/58	0.340	1.6	0.6–4.3			
Y419Src cytoplasm expression (positive/negative)	27/48	0.522	1.3	0.6–2.9			
Y419Src membrane expression (positive/negative)	41/34	0.692	1.2	0.5–2.5			
Y215Src nuclear expression (positive/negative)	22/53	0.590	1.3	0.5–3.0			
Y215Src cytoplasm expression (positive/negative)	30/45	0.007	0.3	0.1–0.8	0.040	0.3	0.1–0.9
Y215Src membrane expression (positive/negative)	3/72	0.771	1.3	0.2–10.1			

Table 3 shows an overview of the ER/PR/HER2-negative patients' characteristics. Each clinical and pathological parameter was correlated to disease specific survival (*P* values). Grade, Bloom and Richardson grade. Histology: ductal = ductal carcinoma; lobular, lobular carcinoma; tubular, tubular carcinoma; others including mucinous, mucoid, and micropapillary carcinoma c-Src, total Src kinase; Y419Src, Src kinase phosphorylated at tyrosine site 419; Y215Src, Src kinase phosphorylated at tyrosine site Y215.

brane.¹ Indeed, in the current study, when Src kinase is activated at the classical site Y419 and located in the cellular membrane, it is associated with shorter disease-specific survival, increasing grade, tumor size, ER negativity, and HER2 positivity. Although our results with Y419 support the role of Src kinase activation, currently described in the literature, we have contradictory results with an alternative phosphorylation site Y215 (Figure 5, A and B). Interestingly, the current study observes poor prognosis associated with membrane expression of phosphorylated Y419Src and good prognosis with cytoplasmic expression of phosphorylated Y215Src. These results suggest that location and not only phosphorylation site influences downstream effectors of Src kinase. Stover et al⁷ report a 50-fold increase in activation of Src kinase associated with Y215Src phosphorylation. In the current study, using clinical specimens, phos-

phorylation at this site is strongly associated with improved survival (high nuclear and cytoplasmic Y215Src expression) and was demonstrated to be independent of other known clinical parameters on multivariate analysis (only seen with high cytoplasmic Y215 Src kinase expression). It is unclear why Y419Src and Y215Src are associated with different outcome measures. These contrasting roles may be due to phosphorylation at Y215 and Y419 residing in different SH domains. Phosphorylation in different domains may result in varying protein configurations, which might enable activation of other downstream signaling pathways. An alternative explanation of these results may be that the antibodies detect phosphorylation of other Src kinase family members (e.g., Lyn, Fck, Yes) in addition or in preference to c-Src, because the phosphorylated regions are highly conserved.⁷

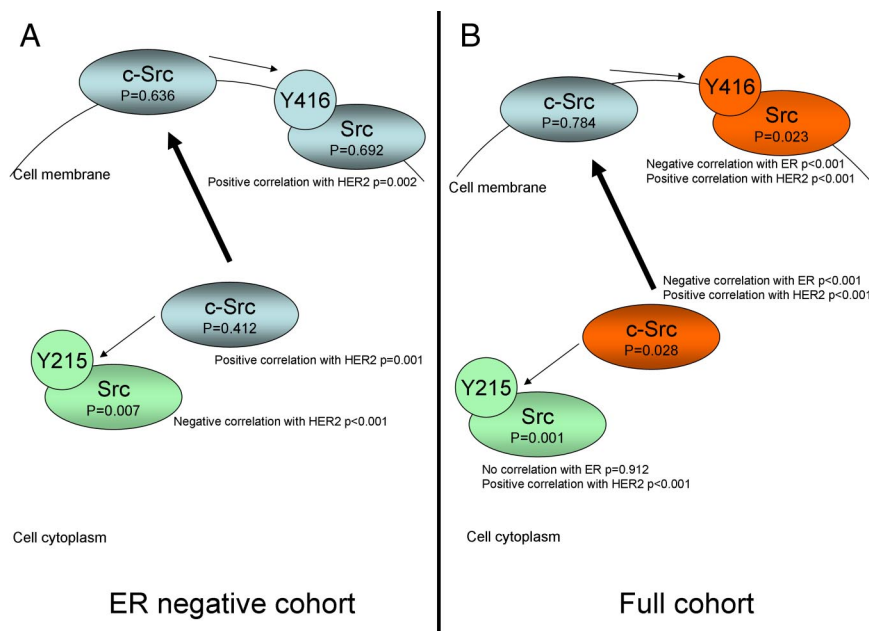


Figure 5. Schematic representation of the interaction between Src kinase, activated Src kinase, estrogen receptor, HER2, and prognosis; highlighting that in the ER-negative cohort membranous Y419Src kinase correlates positively with HER2 while cytoplasmic Y215Src kinase is associated with good prognosis (colored in green) and correlates negatively with HER2 (A). *P* values stated within the ovals represent the association of Src kinase expression with overall survival. Ovals colored in blue were not significantly associated with survival. In the full cohort, cytoplasmic Src kinase and membranous activated Y419Src kinase is associated with poor prognosis (colored in red) and correlates negatively with ER and positively with HER2 status. As in the negative cohort cytoplasmic activated Y215Src kinase is associated with good prognosis, but correlates positively with HER2 status (B).

Activated phosphorylated Y215Src kinase was independent in multivariate analysis (tumor grade, tumor size, axillary lymph node involvement, ER, and HER2 status), providing evidence that Y215Src could be used as a prognostic marker to determine which subgroup of breast cancer patients should not be targeted with Src kinase inhibitors. Although Y215Src was independent on multivariate analysis, there was a positive association observed between cytoplasmic Y215Src kinase expression and HER2 status. These results support the observation that HER2 may phosphorylate Src kinase at Y215.¹⁸ Vadlamudi et al¹⁸ suggested that the interaction with HER2 would result in a more aggressive phenotype of cancer. This is in direct contrast with results obtained in the current study. Further investigations, to determine the underlying molecular interactions between HER2 and Src kinase, are therefore warranted.

Approximately 10 to 15% of breast cancers fall into the receptor negative or triple negative group of breast cancers as defined by the absence of ER, PR, and HER2 receptors. This group of breast tumors are currently the focus of much attention because of their poor prognosis and the lack of an effective treatment target.^{19,20} *In vitro* studies, using gene expression profiling, have identified triple negative breast cancer tumors to be sensitive to dasatinib, a nonreceptor tyrosine kinase inhibitor. A reduction in cell proliferation was demonstrated.¹⁷ When we examined the ER-, PR-, and HER2-negative breast tumors as a representation of the triple-negative group of breast tumors, we found that high cytoplasmic Y215 Src kinase expression resulted again in a significant survival advantage. With Src inhibitors being trialed clinically, it needs to be established which phosphorylation site they target. If subsequent experimental data demonstrates Src kinase inhibitors targeted Y215, these drugs could be potentially harmful to this specific subgroup of patients. Caution should be applied before these drugs are administered.

In conclusion, high expression of cytoplasmic c-Src kinase and membranous-activated Y419Src kinase are associated with a reduction in disease-specific survival. In contrast, activation of cytoplasmic Src kinase at Y215 is associated with improved disease-specific survival, especially in ER-negative patients. Therefore, inhibition of Src phosphorylation via Src inhibitors may possibly be harmful to this particular subgroup. More detailed studies must be conducted to support these findings, especially in view of a large number of Src inhibitors entering clinical trials.

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